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(71)(72) Applicants and Inventors: SPEARS, Colin, P. [US/US]; 3376 Tyrrell Place, Glendale, CA 91206 (US). GUSTAVSSON, Bengt, G. [SE/SE]; Bergsbogatan 29, S-Vastra Frolund (SE). CARLSSON, Goran [SE/SE]; Zazarezagen 33, S-433 75 Partille (SE).

(74) Agents: SCOTT, Watson, T. et al.; Cushman, Darby & Cushman, Eleventh Floor, 1615 L Street, N.W., Washington, DC 20036 (US).

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(54) Title: 5,10-METHYLENE-TETRAHYDROFOLATE AS A MODULATOR OF A CHEMOTHERAPEUTIC AGENT

(57) Abstract

The present invention relates to the compound 5,10-methylene-tetrahydrofolate (CH_2FH_4), and its solution isomer FH_4, therapeutic uses of these compounds, and compositions thereof. CH_2FH_4 and FH_4 strongly modulate the *in vivo* antitumor effects of 5-Fluorouracil.

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5,10-METHYLENE-TETRAHYDROFOLATE AS A MODULATOR OF A CHEMOTHERAPEUTIC AGENT

BACKGROUND OF THE INVENTION

Technical Field

The subject matter of the present invention relates to 5,10-methylene-tetrahydrofolate (CH_2FH_4), therapeutic uses of this compound and compositions thereof. CH_2FH_4 strongly modulates the *in vivo* antitumor effects of 5-Fluorouracil. Furthermore, the present invention additionally relates to a solution isomer of CH_2FH_4, tetrahydrofolate (FH_4), which also strongly modulates the *in vivo* antitumor effects of 5-Fluoruracil.

Background Information

The compound 5-Fluorouracil (5-FU) is possibly the most widely used anticancer drug in the world. In the 1970s and early 1980s, the prevailing opinion among cancer researchers was that the key biochemical lesion caused by 5-FU in tumor cells resulted from the drug's incorporation into RNA (Kufe et al., J. Biol. Chem. 256:9802 (1981) and Glazer et al., Mol. Pharmacol. 21:468 (1982)).

In 1982, using a specifically designed assay of the DNA enzyme, thymidylate synthase (TS) (EC 2.1.1.45), the present inventors established that the therapeutic mechanism of 5-FU against murine colon cancer was complete inhibition of TS or abrogation of TS activity (Spears et al., Cancer Res. 42:450-56 (1982)). In fact, the present inventors were the first to report a clinical correlation between TS level in a patient's cancer after 5-FU treatment and response (Spears et al., Cancer Res. 44:4144-50 (1984)). The finding has been confirmed by several research groups.

TS is the only intracellular source of new ("de novo") thymine synthesis, as the enzyme which catalyzes the methylation of deoxyuridylate to form thymidylate (thymine-2'-deoxyribose-5'-phosphate). Thymine is one of the four main building blocks of DNA, and its occurrence in DNA (vs. its absence in RNA) is the major structural difference between DNA and RNA. Thus, the activity of TS to make new thymidylate and DNA is essential to cell division, tissue regeneration and turnover, and tumor growth. The source of the methyl one-carbon group for synthesis of thymidylate is CH_2FH_4 and its polyglutamates. The mechanism of methyl transfer by TS has recently been reviewed (K.T. Douglas, Medicinal Res. Rev. 7:441-75 (1987)). After initial weak binding of deoxyuridylate to TS, the enzyme catalyzes ring-opening of CH_2FH_4 at the imidazole C11 ring. This may be the rate limiting step overall. The relative stability of tetrahydrofolate within the ternary complex, toward oxidation, suggests that the ring-opening occurs with the substitution at N5, in accordance with formation of an N5-iminium cation species (S.J. Benkovic, Ann. Rev. Biochem., 49:227-51 (1980)). Covalent bonding between the methylene group and the C5-position of deoxyuridylate is accompanied by rapid hydride transfer from the C6-position of the ring-opened CH_2FH_4 so that CH3- is formed on the C6 position of the nucleotide. This leads rapidly to expulsion of the two products from the TS binding site(s), i.e., thymidylate and dihydrofolate. TS is the only enzyme which oxidizes reduced folates to dihydrofolate, which is then converted back to tetrahydrofolate by another enzyme, dihydrofolate reductase. In general, the limiting intracellular factors in this biochemical pathway for making thymine are, in order of increasing scarcity, deoxyuridylate, dihydrofolate

reductase, TS, and then CH_2FH_4. Thus, a decrease in thymidine production through the TS pathway can result from nutritional deficiencies which decrease CH_2FH_4 production (i.e., primary folate deficiency, B12, B6, and other B-vitamin deficiencies which impair folate one-carbon metabolism), or from antimetabolites drugs such as 5-FU or methotrexate. Methotrexate inhibits dihydrofolate reductase, thus blocking the regeneration of tetrahydrofolates from dihydrofolate. 5-FU and other fluorinated pyrimidines (for example, floxuridine, FUDR or trifluoromethylthymidine) block TS activity through formation of the specific metabolite for this effect, fluorodeoxyuridylate (FdUMP), discussed below.

Inhibition of TS activity leads to "thymineless cell death" or "unbalanced cell growth," whereby RNA and protein synthesis, and cell enlargement, occur in the absence of adequate new DNA synthesis (see Goulian et al., Adv. Exp. Med. Biol. 195:89-95 (1986), and refs. therein). In blood cells, such unbalanced cell growth can lead to megaloblastic anemia, macrocytosis, and bone marrow failure.

The mechanism of inhibition of TS by FdUMP has been studied intensively for the past two decades (see Santi et al., Biochem., pp. 8606-13, (1987) and refs. therein). In the absence of CH_2FH_4, FdUMP binds TS extremely weakly. However, in the presence of a large excess of CH_2FH_4, even low levels of FdUMP will bind tightly to TS, by forming inhibitory TS-FdUMP-CH_2FH_4 ternary complexes. In the presence of excess CH_2FH_4, such ternary complexes are stable and no significant TS activity occurs. The molecular basis for the ternary complex is that after CH_2FH_4 ring-opening to form a covalent bond to FdUMP in the TS enzyme pocket (analogous to the

normal reaction with deoxyuridylate), no hydride ion transfer can occur. Thus, no dihydrofolate is formed and the covalently-bonded FdUMP-CH_2FH_4 only leaves the enzyme site with great difficulty, as long as free CH_2FH_4 is present in substantial excess. If the CH_2FH_4 concentration is relatively low, the ternary complex dissociates back to starting products, including free, active TS.

Thus, TS inhibition can occur with only trace amounts of FdUMP in slight excess over TS molecules; however, a specific condition must occur in that 5-10-methylenetetrahydrofolate (CH_2FH_4) (and its polyglutamates) must be present in high concentration. Stated more simply, CH_2FH_4 is like a "glue" that holds the FdUMP onto the TS enzyme and therefore inhibits TS activity. However, CH_2FH_4 is also a powerful growth factor, for promotion of purine, protein, and lipid metabolism, as well as pyrimidine synthesis; thus, CH_2FH_4 administration for the purpose of promotion of TS inhibition by FdUMP may be expected to also increase the degree of "unbalanced cell growth."

CH_2FH_4 is a normal intracellular metabolite of the B-vitamin, folic acid, for use in thymidylate synthesis by TS. The same is true with respect to the polyglutamates of CH_2FH_4. However, CH_2FH_4 is also used by several other enzymes including CH_2FH_4 reductase (EC 1.1.99.15), serine hydroxymethylase (EC 2.1.2.1), and C1-tetrahydrofolate synthase and CH_2FH_4 dehydrogenase (EC 1.5.1.5). These interconversions using CH_2FH_4 are essential for purine synthesis, amino acid synthesis (i.e., serine and methionine), and lipid metabolism through the re-methylation of methionine. Thus, CH_2FH_4 is located at a metabolic branch point as a substrate for at least 4 different enzymes (Green et al., Biochem. 27:8014-22, (1988), S.J. Benkovic, Ann.

Rev. Biochem. 49:227-51 (1980) and Schirch et al., Arch. Biochem. Biophys. 269:317-80 (1989)). This explains the fact that intracellular CH_2FH_4 is normally present in low concentrations, below 1.0 micromolar. Recent measurements have shown that intracellular CH_2FH_4 levels are typically low, and virtually always lower than tetrahydrofolate, using the bacterial L. Casei TS-[3H]FdUMP ligand binding assay (Priest et al., Cancer Res. 48:3398-3404 (1988), and refs. therein). The present inventors have modified this assay (Adv. Exp. Med. Biol. 244:98-104 (1988) and Invest. New Drugs 7:27-36 (1989)) and reported relatively low levels of CH_2FH_4 (much below 1.0 micromolar) in patients' cancer biopsy specimens despite administration of high doses of leucovorin (LV) (Proc. Am. Soc. Clin. Oncol. 8:69 (1989)); furthermore, these observations of the present inventors led to administration of the amino acid, L-serine, to patients in an attempt to convert the tetrahydrofolates (in various polyglutamate forms, present in large excess) to CH_2FH_4 (and polyglutamates). These results have suggested that increased FH_4, rather than CH_2FH_4, may be therapeutic. The inventors have recently published the only comparative data that exist for the different major intracellular one-carbon forms of folates (Biochem. Pharmacol. 38: 2985-93 (1989)), showing that of all of these, CH_2FH_4 (at least, as the monoglutamate) is the best folate form for formation of TS-FdUMP-folate ternary complexes, and that a concentration of CH_2FH_4 in excess of 1.0 micromolar is desirable for this effect. CH_2FH_4 was found to be four times stronger than the next best folate, tetrahydrofolate, and about 100 times stronger than LV.

Leucovorin (referred to as LV, or folinic acid) is (6R,S)-5-formyl-tetrahydrofolate and has

been available commercially for decades for the treatment of folic acid (the B-vitamin) deficiency states (The Pharmacologic Basis of Therapeutics, 4th ed. (Goodman et al., eds.) The MacMillan Co., Toronto, pp. 1431-44 (1970)). In 1982, the first clinical reports of the usefulness of LV as a modulator of 5-FU in cancer treatment appeared. (Machover et al., Cancer Treat. Rep. 66:1803-07 (1982)). LV addition to 5-FU appeared to approximately double response rates in patients with gastrointestinal cancers. This result was confirmed in several subsequent studies. (For an extensive review, see Grem et al., Cancer Treat. Rep. 71:1249-64 (1987)). Currently, LV addition to 5-FU therapy is community standard practice in the United States.

The mechanism of leucovorin (LV or folinic acid) improvement in the antitumor therapy of 5-FU and floxuridine (FUDR) has been shown in several studies to be due to improved TS inhibition associated with increased intracellular (6R)-CH_2FH_4 and (6S)-tetrahydrofolates. However, LV appears to be only partially effective in the goal of promoting complete TS inhibition by FdUMP in vivo. For an in vitro example, researchers have shown that TS inhibition after 5-FU, while improved by LV, was still clearly incomplete (Keyomarsi et al., J. Biol. Chem. 263:14402-09 (1988)). In part, this may have been related to saturation of obtainable summed pools of CH_2FH_4 + tetrahydrofolate at about a 5-fold increase over baseline at 30 hr LV exposure. Thus, maximum synergy of LV was obtained at less than 1.0 micromolar exposure, with no further improvement at higher concentrations although human plasma folates (LV and methyltetrahydrofolate, MTHF) are higher than this after high-dose LV administration (Doroshow et al., NCI Monogr. 5:171-74 (1987)). A related observation may be that addition of high-

dose folic acid (140 mg/m2) to 5-FU therapy appears to be associated with an increase in toxicity without improved response rates (Asbury et al, Am. J. Clin. Oncol. 10:47-49 (1987)).

In fact, decreasing synergy has been shown for LV addition to FUDR at concentrations above 0.5 micromolar, when the colon cancer cells were previously folate-deficient (Davis et al., Mol. Pharmacol. 35:422-27 (1989)). Also, others have shown in vivo in mice that expansion of breast tumor CH_2FH_4 pools was a maximum of less than two-fold over baseline despite massive LV dosing (180 mg/kg x 8 over 48 hr) (Wright et al., Cancer Res. 49:2592-96 (1989)). These observations are mirrored in recent clinical trials comparing the therapeutic outcome in colon cancer, in which low-dose LV (20 mg per square meter) was more effective than high-dose LV (200 mg per square meter) in terms of both tumor response rate and patient survival (Poon et al., J. Clin. Oncol. 7:1407-18 (1989)). The lack of effectiveness of high-dose LV in promoting complete TS inhibition was suggested by researchers based on tumor biopsy analyses in breast cancer patients: LV increased TS inhibition from an average of 30 $\pm$ 13 to 71 $\pm$ 14 %, with responding patients showing the higher percentages of TS inhibition than non-responders (Swain et al., (J. Clin. Oncol. 7:890-99 (1989)).

In view of the above, the present inventors realized the potential of the direct administration of CH_2FH_4 to patients receiving 5-FU, as such a course of action would maximize TS inhibition.

The desirability and ability to use CH_2FH_4 in the method of the present invention have never been obvious for various reasons.

For example, CH_2FH_4 as a compound in solution has enjoyed a general reputation of being

extremely unstable. (Temple et al., "Chemical and Physical Properties of Folic Acid and Reduced Derivatives," In Folates and Pterins (Blakely et al., eds.), Vol. 1, pp. 61-63 (1984) and Wright et al., Cancer Res. 49:2592-96 (1989)). In solution, it is generally known to exist in equilibrium with FH_4, requiring excess formaldehyde to favor the equilibrium toward CH_2FH_4.

Under anaerobic conditions, such as made possible for clinical administration of CH_2FH_4 by a closed, delivery system (U.S.Patent 4,564,054), powdered tetrahydrofolate is stable even at room temperature, for a year or more (Caldwell et al., Prep. Biochem. 3:323-26 (1973)).

Additionally, published data on the clinical tissue levels of CH_2FH_4 in patients have been limited, and it is well known that LV can be given in gram-size doses (Grem, et al., supra.). LV is an extremely powerful folate (B-vitamin) that is one-hundred times stronger than folic acid in correcting nutritional folate deficiency. As little as 1.0 mg of LV will correct folate deficiency as a single dose (The Pharmacological Basis of Therapeutics, supra.). Thus, it is logical to assume that tumor CH_2FH_4 levels might reach saturation levels from high dose LV.

Finally, it appears that no published studies exist on the toxicological aspects of CH_2FH_4. More specifically, there seems to be no available published work on either in vitro or in vivo effects of direct exposure of living cells to CH_2FH_4.

Thus, in view of the structural properties of CH_2FH_4 as well as the lack of information regarding the effects of CH_2FH_4, the present invention is quite remarkable. CH_2FH_4 is utilized to

potentiate or modulate the antitumor effects of the chemotherapeutic agent 5-FU.

L.R. Hughes (Eur. Pat. Appl. EP 284,3380 and Chem. Abstr. 110:95789 (1989)) has described a novel folate analog as a TS inhibitor and antitumor agent. However, the discovery is clearly radically different from the present invention. The analog does not occur naturally, is absent two nitrogen atoms, is not reduced, and has a reactive propargyl group attached to the glutamate moiety. Also, no mention is made of 5-FU.

Interleukin-2 has been proposed as a modulator of tetrahydrobiopterin (US Patent 4,752,573); however, interleukin-2 is an oligopeptide having no resemblance to leucovorin, and no claim for TS inhibition or interaction with 5-FU is made.

A patent for radiolabeled assay of folates (US Patent 4,136,159) has no therapeutic pharmaceutical intent, and makes no mention of TS inhibition.

Various patents exist for other, unnatural folate analogs, including quinazolines and dideazatetrahydrofolates as inhibitors of enzymes such as folylpolyglutamyl synthetase (e.g., see Chem. Abstr. 110: P39366p (1989)). However, these are unnatural analogs which have distinct chemical, structural differences from CH_2FH_4.

The European patent application (EP 266,042) of Wood et al. describes a process for separation of diasteriomers of LV, as well as (6R)- and (6S)-tetrahydrofolates. No use of CH_2FH_4 as a potentiator of TS inhibition by FdUMP (and thus 5-FU and other fluoropyrimidines) is claimed in the document.

All U.S. patents and publications referred to herein are hereby incorporated by reference.

SUMMARY OF THE INVENTION

The present invention relates to the compound CH_2FH4 and its solution isomer FH_4, therapeutic uses of these compounds, and compositions thereof. CH_2FH_4 and FH_4 strongly potentiate the antitumor or TS-inhibitory effects of 5-FU.

More specifically, the present invention includes a method of inhibiting the growth of a tumor in a patient comprising administering to said patient an amount of parent CH_2FH_4 or FH_4 and 5-FU sufficient to effect said growth inhibition. The CH_2FH_4 or FH_4 may be administered concurrently with 5-FU, or prior to the administration of 5-FU. In the latter case, the CH_2FH_4 or FH_4 is administered 6-24 hours, or preferably 1-3 hours, before the administration of the 5-FU.

The CH_2FH_4 or FH_4 may also be administered after the administration of 5-FU in which case the CH_2FH_4 or FH_4 compound is administered 1-10 days, or preferably 1-6 hours, after the 5-FU administration.

Furthermore, the CH_2FH_4 or FH_4 solution may be administered either intravenously, intraarterially, or intraperitoneally, and in a dosage of 5-500 mg/m^2 (body surface area). Preferably, it may be administered in a dosage of 20-200 mg/m^2 (body surface area). The CH_2FH_4 or FH_4 solution may also be administered orally or topically as a 0.5% cream under an occlusive dressing.

If it is administered intravenously, such as through a central venous catheter, the CH_2FH_4 or FH_4 solution may be given in a dosage of 5-500 mg/m^2 (body surface area), or preferably 20-200 mg/m^2, every 4-6 hours, once daily, or once weekly or as a

continuous infusion of 20-200 mg/m²/week. Additionally, if it is administered every 4-6 hours, the CH_2FH_4 or FH_4 solution may be administered prior to, or subsequent to, the administration of 5-FU.

The CH_2FH_4 or FH_4 may be administered as the 6R, 6S, or as a mixture of the 6R and 6S enantiomers (diastereomers).

Also, if the CH_2FH_4 or FH_4 is administered in an alkaline vehicle, the concentration of the CH_2FH_4 or FH_4 is from 0.1 to 20 mg/ml whereas if the compound is administered in physiologic saline, the concentration is from 0.1 to 10 mg/ml.

Furthermore, the present invention includes a method of using CH_2FH_4 or FH_4 in order reduce the toxicity of an anti-folate drug which has been administered to a patient. Examples of anti-folate drugs include methotrexate, trimetrexate, nitrous oxide, and dideoxytetrahydrofolic acid.

The present invention also includes a method of treating folate deficiency states by the administration of CH_2FH_4 or FH_4.

Moreover, the present invention also includes a method of treating B12- and B6-refractory anemias whereby CH_2FH_4 or FH_4 is administered in an amount sufficient to effect said treatment.

Furthermore, the present invention also includes a composition containing CH_2FH_4 or FH_4 and 5-FU, as well as a pharmaceutically active carrier. The composition may also contain a stabilizing agent such as an ascorbate salt, or glutathione. The composition may also contain free formaldehyde.

Additionally, the present invention also includes a composition containing CH_2FH_4 or FH_4 and a compound which is metabolized to FdUMP, as well as a pharmaceutically active carrier. Examples of

compounds which can be metabolized to FdUMP include floxuridine (FUDR), ftorafur (tegafur), and 5'-deoxyfluorouridine (Doxifluridine®). The composition may also contain a stabilizing agent, such as an ascorbate salt, or glutathione. Formaldehyde may also be present in the composition.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 represents the effect of CH_2FH_4 ("$CH_2H_4PteGlu_n$") on TS inhibition in 5-FU-resistant colon cancer cells (from tumor 51) after the administration of 5-FU ("FUra").

Figure 2 represents the structure of (6R,S)-methylene-tetrahydrofolic acid (or CH_2FH_4) and the configuration of the natural (6R)-CH_2FH4 enantiomer (diastereomer) (Poe et al., Biochem. 18:5528 (1979) and Kalbermatten et al., Helv. Chim. Acta 64:2633 (1981)).

Figure 3 represents the structure of tetrahydrofolic acid or FH_4, the predominant form at concentrations of less than 1 mM.

Figure 4 shows the results of TS-[^{3}H]FdUMP-folate binding assay of CH_2FH_4 as a function of concentration of the folate in 0.2 M Tris buffer, pH 7.4, with and without formaldehyde (CH_2O), 6 mM, addition.

DETAILED DESCRIPTION OF THE INVENTION

One embodiment of the present invention relates to the use of CH_2FH_4 as a modulator of 5-FU in cancer chemotherapy. CH_2FH_4 as well as FH_4, increase response rates to 5-FU as a result of increasing the inhibition of TS by the 5-FU metabolite, FdUMP, in tumors. Thus, CH_2FH_4 can be used to inhibit the growth of tumors when used in combination with 5-FU, or with other drugs which are

metabolized to FdUMP including floxuridine (FUDR), ftorafur (tegafur), and Doxifluridine® (5'-deoxyfluorouridine).

The mechanism of action of CH_2FH_4 is promotion of TS inhibition by FdUMP in fluoropyrimidine-treated tumors, which can occur by increasing the rate of formation and stability of TS-FdUMP-CH_2FH_4 and TS-FdUMP FH_4 ternary complexes. Administration of CH_2FH_4 in doses ranging from 5-500 mg/m^2 (body surface area), or preferably 20-200 mg/m^2, will result in expansion of intracellular pools of both CH_2FH_4 and FH_4 as monoglutamates. These are the best two folate forms as substrates for polyglutamation, the major intracellular forms for retention of folates, as well as for direct binding to TS-FdUMP complexes. One carbon exchange between endogenous CH_2FH_4-polyglutamates and tetrahydofolate-monoglutamate resulting from CH_2FH_4 administration, as suggested in Tables II and III, would indicate that the optimal times for bolus 5-FU administration are concurrently or at several hours after bolus I.V. CH_2FH_4 administration and thus after maximum polyglutamation. CH_2FH_4 may also be administered after 5-FU is given or as a protracted, continuous infusion.

More specifically, CH_2FH_4 may be administered 6-24 hours, or preferably, 1-3 hours, prior to the administration of 5-FU. CH_2FH_4 can also be administered 1-10 days, or preferably 1-6 hours, subsequent to the administration of 5-FU.

Polyglutamation of folates causes retention within the cell, and typically also accelerates rates of enzyme processing of one-carbon interconversions of folates (Schirch et al., Arch. Biochem. Biophys. 269:371-80 (1989), Green et al., Biochem. 27:8014-22, 1988). Current data would suggest that polyglutamation of FH_4 and CH_2FH_4 will

promote TS-FdUMP-folate inhibitory ternary complex formation to a greater extent than promotion of the normal enzymic reaction with deoxyuridylate (Houghton et al., Cancer Res. 48:3062-69 (1988)). Since polyglutamates may form TS-FdUMP-folate ternary complexes as much as 50-fold more tightly than parent monoglutamates, an objective of folate addition to fluoropyrimidine therapy could also include formation of TS-FdUMP-tetrahydrofolates, which would also be strongly inhibitory. In addition, a role for the unnatural enantiomers (diastereomers at the pterin C6- position), such as polyglutamates of (6S)-CH_2FH_4 or (6R)-tetrahydrofolate, in TS inhibition by forming TS-deoxyuridylate-folate or TS-FdUMP-folate ternary complexes, potentially could be a factor (Kisliuk et al., Biochem. 20:929-34 (1981)) in the TS inhibition observed with CH_2FH_4 administration in vivo (Tables I, II, and III; Fig. 1).

The potentiation of TS inhibition by low levels of FdUMP may be expected to last only a few hours unless polyglutamation of the CH_2FH_4 and FH_4 occurs thereby creating more powerful TS-FdUMP binders than the parent monoglutamate. Thus, CH_2FH_4 dosing requirements may be as frequent as every 4-6 hrs., once daily, or as infrequent as once weekly.

In one embodiment of the present invention, CH_2FH_4 can be administered by intermittent (e.g., daily) bolus dosing in patients who have central venous catheters. Such patients could self-administer the CH_2FH_4 (using a means for ensuring the stability of the formulation to oxidation) and would also be candidates for administration of CH_2FH_4 by continuous, intravenous protracted infusion. The 5-FU infusion would be expected to produce low levels of FdUMP in tumors. Low FdUMP levels would be expected to be associated with relatively poor TS

inhibition unless CH_2FH_4 levels were very high. FH_4, free of formaldehyde as a stabilizer may also be administered in the same manner.

An ameliorating factor to consider may be that chronic TS inhibition, albeit incomplete, would be expected to cause slight increases in CH_2FH_4 levels because of lowered consumption of CH_2FH_4 in the natural TS mechanism so that pharmaceutical CH_2FH_4 in this setting might be more efficient.

Other embodiments include the addition of CH_2FH_4 at late times after bolus intravenous 5-FU infusion (e.g., at 6 hours in the daily 25 (monthly) Schedule, or at days 4, 5 and 6 on the biweekly bolus schedule.)

In addition to being administered intravenously, CH_2FH_4 may also be administered intraarterially or intraperitoneally, also in a dosage of 5-500 mg/m^2, or preferably, in a dosage of 20-200 mg/m^2. However, CH_2FH_4 may also be administered topically as a 0.5% cream under an occlusive dressing.

Another embodiment of the present invention comprises a composition containing CH_2FH_4 as well as 5-FU. The composition also contains a pharmaceutically active carrier, and may also contain formaldehyde in excess as a stabilizer.

A further embodiment of the present invention includes a composition containing CH_2FH_4 and one or more other drugs which can be metabolized to FdUMP. The composition may contain a pharmaceutically active carrier, and may also contain formaldehyde in excess as a stabilizer.

It should be noted that FH_4, free of formaldehyde, can replace the use of CH_2FH_4 in each of the above embodiments.

Because reduced folates are rapidly interconvertible according to their one-carbon

states, it may be anticipated that the clinical tolerance for CH_2FH_4 or FH_4 will be similar to that of LV and 5-methyl-tetrahydrofolate (MTHF), the latter of which is the predominant blood transport form of folates.

Also, tetrahydrofolate, and possibly CH_2FH_4, have recently been reported as accumulating to low but significant (i.e., less than 20 micromolar) concentrations in human plasma after LV administration to human subjects (Bunni et al., Cancer Chemother. Pharmacol. 23:353-57 (1989)).

Thus, it can be anticipated that the dose tolerance for CH_2FH_4 or FH_4 in humans is similar to the reported experiences with LV and methyltetrahydrofolate (MTHF) (both of which are given as a mixtures of enantiomers). Specifically, an upper limit of 500 mg per square meter body surface area would be expected to be therapeutically effective. The lowest effective dose may possibly be more powerful than either LV or MTHF, and thus could be as low as 5 mg per square meter body surface area in a single dose. A dosage of 20-200 mg/m^2 (body surface area) is preferred.

Based on previous studies of the toxicology of folates (LV, MTHF and folic acid) combined with 5-FU and fluorodeoxyuridine, the LD50 in rats would be expected to be above 150 mg/kg i.v. (single bolus) with regard to CH_2FH4 or FH_4, and may be expected to cause convulsions in such high doses (Bartosek et al., Chemioterapia Oncologica 2(4): 85-98 (Dec. Supp, 1987)).

The pH of the CH_2FH_4/FH_4 solution which is to be injected, may range from slightly acidic to slightly alkaline. 5-FU up to 50 mg/mL in alkaline media may be present, analogous to the practice of formulation of 5-FU and LV in the same solution (e.g., Trave et al., J. Clin. Oncol. 6:1184-91

(1988)). Furthermore, the concentration for injection may be as high as 100 mg/10 mL, preferably from 0.1 to 20 mg/ml, in alkaline vehicles. The concentration may also be as high as 100 mg/20 mL, preferably from .1 to 10 mg/ml, in physiologic, normal saline. At concentrations less than 1 mM in initial CH_2FH_4 concentrations, the predominant form in solution is FH_4 (i.e., the dilution of CH_2FH_4 in aqueous solution shifts the equilibrium between FH_4 and CH_2FH_4 towards FH_4, regardless of pH, O_2 tension, or the presence of reducing agents).

Ascorbate salts may be present as stabilizers (e.g., 1% w/v as the salt at neutral or slightly alkaline pH). Other reducing substances may also be used as stabilizers, for example, reduced glutathione.

Free formaldehyde (CH_2O) may also be present in concentrations up to 10 mM. However, the dosage must be adjusted for formaldehyde toxicity. The formulation may be made directly from (6R,S)-FH_4 powder, alternatively. In this case, formulations would be checked and controlled for the degree of spontaneous condensation of formaldehyde from ambient air to form CH_2FH_4. The oral LDLo (or lowest lethal dose) of CH_2O in humans has been reported to be 36 mg/kg (Registry of Toxic Effects of Chemical Substances, US DHHS, PHS, CDC, NIOSH, Vol. 1, p. 822 (1980)). The pure (6R)CH_2FH_4 or (6S)FH_4 enantiomer may also be utilized, free of the non-TS-binding, unnatural (6S)CH_2FH_4 or (6S)FH_4 enantiomer, respectively. Enantiomer separation is obtainable by chiral column or DEAE column preparative isolation (Kaufman et al., J. Biol. Chem. 238:1498-1500 (1963)).

A major advantage of CH_2FH_4 over FH_4 as the parent powdered material is the protection against oxidation, referred to above, which protection would

therefore be greater with concentrated versus dilute (e.g., < 0.5 mM) concentration, in the absence of a mechanism for excluding air during reconstitution and administration (as provided by the Protector device).

It appears that direct administration of CH_2FH_4 or FH_4, either as the mixture of 6R and 6S diastereomers (enantiomers), the unnatural 6S-CH_2FH_4, or the natural 6R-CH_2FH_4 alone (or their FH_4 solution equilibrium products) can overcome some of the disadvantages of LV described above. That is, CH_2FH_4 addition to 5-FU can lead to greater tetrahydrofolate and CH_2FH_4 elevations intracellularly than LV or MTHF (which both require one carbon activation), and consequently show more profound synergism on TS inhibition by FdUMP.

The applications for CH_2FH_4 or FH_4 are quite significant and far-reaching. For example, antitumor uses of CH_2FH_4 or FH_4, combined with TS-inhibitory fluoropyrimidines include: 1) addition to Platinol/5-FU infusion therapy in head and neck cancer and other epidermoid cancers, 2) addition to combination cyclophosphamide/doxorubicin/5-FU in breast cancer 3) addition to topical Efudex® (5-FU) cream under an air-free occlusive dressing for skin conditions (for example benign keratoses, keratoacanthomas, verrucae, premalignant keratoses, in situ cancer and invasive superficial malignancies amenable to topical therapy). Furthermore, CH_2FH_4 or FH_4 can also be applied to those cancer types in which 5-FU and floxuridine are typically combined with LV, such as in colon, rectal and pancreatic carcinomas.

CH_2FH_4 or FH_4 can also be utilized with respect to non-malignancy related conditions. For example, CH_2FH_4 or FH_4 can be used with respect to B12- and B6-refractory anemias which are not

responsive to LV. CH_2FH_4 or FH_4 can also be used to treat folate deficiencies. Furthermore, CH_2FH_4 and FH_4 can also be used for the potentiation (selective rescue of the host patient) of the TS inhibitory mechanism of antibacterial action of nucleotide analogs.

Additionally, CH_2FH_4 or FH_4 can be utilized to reduce the toxicity of anti-folate drug which have been administered to patients. Such anti-folate drugs include, for example, methotrexate, trimetrexate, nitrous oxide, and dideoxytetrahydrofolic acid.

As a rescue agent following methotrexate, CH_2FH_4 or FH_4 may be more specific than the presently used LV (or MTHF) since CH_2FH_4 would require less (or no) metabolic activation in the case of FH_4 to provide for purine, pyrimidine, and the amino acid synthetic requirements normally met by intracellular folates. CH_2FH_4 could also therefore become useful in rescue of the host in the trimetrexate treatment of Pneumocystis carinii infections of immunosuppressed patients (i.e., AIDS patients).

The present invention can be illustrated by the use of the following non-limiting examples.

Example 1

Synthesis of CH_2FH_4 as a Low-Formaldehyde Material

Preparation of (6R,S)-CH_2FH_4:

CH_2FH_4 as the equal mixture of diastereomers (optical isomers or enantiomers at the C6-position; both diastereomers are of the natural L-configuration at the alpha-carbon position of the glutamate moiety) was prepared from (6R,S)-tetrahydrofolic acid, commercially available from Sigma, in the examples described below. The method of synthesis has been described previously (C.P.

Spears and B. Gustavsson, Adv. Exp. Med. Biol. 244:98-104 (1988)). To (6R,S)-tetrahydrofolate powder, (100 mg) is added 360 µL of 1.0 M Na Ascorbate, pH 6.5, 68 µL of 37% (w/w) formaldehyde (CH_2O), and 16 mL phosphate buffer, pH 7.0. A 10-min room temperature incubation allows completion of formation of (6R,S)-CH_2FH_4. This material is applied to a DEAE-cellulose column using a modification of a well-known procedure (Kaufman et al., J. Biol. Chem. 238:1498-1500 (1963)). A step elution with NH_4HCO_3 buffers of increasing concentration and pH, leads to isolation of CH_2FH_4 in the last pooled fraction. This material does not contain free formaldehyde as assayed Colorimetrically by toluene extraction of dimedone (methone)-trapped [11-^{14}C] CH_2FH_4, prepared with [^{14}C]CH_2O as described previously (Moran et al. Proc. Natl. Acad. Sci. USA 76:1456-60, 1979). Phosphate buffers and TEAE-cellulose can also be used in the procedure of Kaufman, which gives both enantiomers of CH_2FH_4 in the same peak; however, if potassium bicarbonate buffer is used, a separation of the enantiomers is effected, with the biologically active, natural-configuration, (6R)-CH_2FH_4 peak eluting after the (6S)-CH_2FH_4 peak. The amount of formaldehyde (as methylene) in the product may, in fact, be even less than stoichiometric with tetrahydrofolate (Horwitz et al, J. Med. Chem. 12:49-51 (1969)). The amount of (6R)-CH_2FH_4 in the preparations is checked by one or more of the three following methods. (1) Spectrophotometrically, by use of this material as the limiting substrate in a TS assay with L.Casei enzyme, as described by Daron et al. (J.Biol.Chem. 253:940-45 (1978); (2) ligand binding assay using [6-3H]FdUMP and L.Casei TS described by the inventors (Adv. Exp. Med. Biol. 244:98-104, 1988); and by absorbance at 294 nm on HPLC (Lu et al., Biochem. 23:6870-75 (1984)).

Column-isolated CH_2FH_4, whether racemic in 6R- and 6S-forms or as the 6R-form alone in solution can be stored under argon at -80°C for up to a year without decomposition (Bruice, et al. Biochem. 21: 6703-09 (1982)). Alternatively, solutions of CH_2FH_4 after column isolation can be lyophilized to powder and stored under nitrogen in sealed glass ampoules. Various ratios of formaldehyde to CH_2FH_4 can be used, from less than stoichiometric, as described above, including no formaldehylde (either bound as methylene, or free) to a 2- to 4-fold or more excess (Bruice, et al., Biochem. 21:6703-07, (1982)). The use of 2-mercaptoethanol or other reduced thiols has been advocated by some workers, but is unnecessary and may cause minimal interference (S.F. Zakrewski, J.Biol.Chem. 241:2957-961 (1966) and Kallen et al. J.Biol.Chem. 241:5845-50 (1966)) in condensation of CH_2O with tetrahydrofolate.

Alternative methods for synthesis and purification of (6R,S)-CH_2FH_4 are reviewed by C. Temple, Jr. and J.A. Montgomery, In: Folates and Pterins (R.L. Blakley and S.J. Benkovic, eds.), vol. 1, Chemistry and Biochemistry of Folates, John Wiley & Sons, New York, pp.61-120 (1984). This includes use of (6R,S)5-formyltetrahydrofolate (LV), which is commercially available in bulk quantities, and is converted to the 5,10-methenyl-tetrahydrofolate by acidic conditions. The latter compound then can yield CH_2FH_4 by reduction with borohydride in DMSO and pyridine (Farina et al., J. Am. Chem. Soc. 95:5409 (1973)).

Preparation of (6R)-CH_2FH_4:

The naturally-occurring diastereomer (enantiomer) of CH_2FH_4, (6R)-CH_2FH_4, can be prepared by a number of methods, including that of Kaufman et al. as described in the foregoing section, using

TEAE-cellulose elution by bicarbonate. Commercially-available folic acid reduced to dihydrofolate using hydrosulfite (Mathews et al. J.Biol.Chem. 235:3304-08, (1960)) or dithionite (R.L. Blakley, Nature 188:231-32, (1960)) is used as a substrate for purified dihydrofolate reductase in the present of NADPH (e.g., see M. Poe et al, Biochem. 18:5527-30 (1979)). Formation of (6S)-tetrahydrofolate (which is the natural diastereomer) is readily followed at 294 nm. Purification is then done by chromatography (e.g., S.F. Zakrewski and A.M. Sansone, Methods Enzymol. 18B:728-31, 1971), followed by lyophilization to powder and storage under nitrogen or argon in sealed glass vials.

An additional approach is reduction of dihydrofolic acid by dihydrofolate reductase in the presence of formaldehyde (Horne et al., Methods Enzymol.66:545ff (1980)), followed by column isolation, which avoids the need for a separate CH_2O step after (6S)-tetrahydrofolate isolation. In these preparations, ascorbate is typically present (e.g., 0.1M) as an antioxidant. Synthesis of the unnatural (6R)-CH_3FH_4 isomer has been described, by selective enzymic conversion of (6R)-CH_3FH_4 to dihydrofolate, which is easily separated by column chromatography (Anal. Biochem., Vol. 154, pp 516-24 (1986)). The isomeric solution of (6S)-FH_4 is obtained by dilution to less than .5 mM.

Stability of CH_3FH_4:

Solutions of CH_3FH_4, as well as the powder, are unstable in the presence of oxygen, with oxygen degradation being catalyzed by light, acid, base, and heavy metals (R.G. Kallen, Methods Enzymol.183:705ff, 1971). CH_3FH4 is somewhat more stable than FH_4, as are the major N5-substituted tetrahydrofolates; FH_4 solutions can undergo 90%

degradation in 4.1 hr when exposed to air (discussed in C. Temple, Jr., and J.A. Montgomery, supra. However, tetrahydrofolate is completely stable under anaerobic conditions Caldwell et al., Prep. Biochem. 3:323-26 (1973).

Thus, a method for air-free reconstruction of CH_3FH_4 or FH_4 powder (in vacuum, or under nitrogen or argon in air-tight ampoules), or fresh handling of column-isolated CH_3FH_4 or FH_4, is required to ensure the stability of CH_3FH_4 as a pharmaceutical with accurate dosing. The invention of Gustavsson, one of the present inventors, (U.S. Patent 4,564,054) referred to as the Protector device, affords such a method. The Protector invention is not generally known, since it is marketed as a method for prevention of aerolization of mutagenic/toxic cancer chemotherapy agents, however, it is equally useful for air-free reconstitution, dosing, and i.v. administration of drug solutions to patients. The Protector is suitable for handling all anticipated dose ranges and concentrations of CH_3FH_4, with the volume for dosing limited only by the syringe size. Vehicles for reconstitution of CH_3FH_4 or FH_4 powder include 5% dextrose, normal (0.89% w/v) saline, 5-FU solutions, and sterile water, (which may or may not be de-aerated for removal of dissolved oxygen prior to use in reconstitution of CH_3FH_4 or FH_4 powder, depending on the presence in the formulation of antioxidant stabilizers such as ascorbate). The Protector may be modified to use semi-opaque materials, such as brown plastic, to reduce transmission of ambient light.

Example 2

CH$_2$FH$_4$ USE WITH 5-FU IN MURINE COLON CARCINOMA CA51

(6R,S)-CH$_2$FH$_4$ was prepared by the DEAE-cellulose column procedure, described above, using step-elution of the material as previously reported for purification of nucleotides (Moran et al., Proc. Natl. Aca. Sci. USA 76:1456-60 (1979)). To twenty micromoles of (6R,S)-FH$_4$ (Sigma) were added 62.5 ul of 1.0 M Na Ascorbate, pH 6.5, 2.7 ul of 37% formaldehyde stock, and 0.6 mL of 5 mM phosphate buffer, pH 7.0. Because of the high formaldehyde, this solution was over 2 mM in CH$_2$FH$_4$, with less FH$_4$ present as the solution isomer. After 20 min at room temperature, this solution was applied to a 1 x 3-cm DEAE-cellulose column; in the last step, the 500 mM NH4HCO3 (pH 8.0) fraction (30 mL) was pooled, lyophilized to dryness, and stored under vacuum in glass ampoules. Spectrophotometric assay of powder reconstituted in phosphate-buffered-saline showed a concentration of (6R)-CH$_2$FH$_4$ in this solution of 2.4 mM; prior assay by L. casei TS-[3H]FdUMP-folate ternary complex formation gave a concentration of 2.5 mM.

On the day of reconstituting the above CH$_2$FH$_4$, mice bearing subcutaneous murine colon carcinoma Tumor 51 were administered intraperitoneal (i.p.) 5-FU, with or without concomitant i.p. CH$_2$FH$_4$ by separate injection. The 5-FU was given at a dose of 1.6 mg per mouse, about 80 mg/kg. The CH$_2$FH$_4$ was given at a dose of 0.5 mL of the 2.4 mM material (1.2 mmole/mouse), above. The in vivo methodologies were essentially as had previously been described (C.P. Spears, et al., Cancer Res.42:450-56 (1982)). In contrast, however, to the extensive prior experience of the present inventors with this 5-FU-

resistant tumor line, which always had shown significant FdUMP-titratable free TS levels, the tumors of mice receiving concomitant CH_2FH_4 showed abrogation of TS activity (Table I and Figure 1). The free TS levels of the 5-FU-only treated mice were comparable to the previous observations of the inventors in this line, and at the 1.0 pmol/g level of TS activity was sufficient to support thymidylate synthesis required for tumor growth (C.P. Spears, Exerpta. Med. Int. Congr. Series 647:12-19, (1984)). The levels of apparent free TS in tumors of mice receiving CH_2FH_4 concomitant with 5-FU were at, or below, that level due to exchange-labeling of endogenous TS-FdUMP-folate ternary complexes in the cytosolic extracts. Stated otherwise, the average ± S.D. apparent TS value of 0.42 ± 0.20 pmol/g for the 5 tumors of the 5-FU + CH_2FH_4 treatment group when corrected downward for labeling of endogenous FdUMP-inhibited enzyme by a minimum correction factor of 5% (Spears and Gustavsson, Adv. Exp. Med. Biol. 244:98-104, (1988)) equates with zero detectable TS activity. This is exactly the qualitative difference between sensitivity and resistance to 5-FU previously established (see Spears et al., Cancer Res. 42:450-52 (1982)). An additional observation was that in the Tumor 51 specimens from mice receiving CH_2FH_4 concomitant with 5-FU was that the pre-incubation dissociation condition, which had previously been routinely used for regenerating all TS in the free form, was completely unable to regenerate free TS, in contrast to the more normal findings in the 5-FU-only exposed tumors. This is strongly suggestive that CH_2FH_4 administration raised concentrations of tumor CH_2FH_4 and FH_4, so high, that even after large dilution into the assays the concentrations were still above those that could

spontaneously oxidize to lower levels permitting in vitro ternary complex dissociation.

The results obtained from Example 2 are shown in Figure 1, and in Table I.

TABLE I

TS INHIBITION IN MURINE TUMOR CA51 AFTER 5-FU[a] EFFECT OF CO-ADMINISTRATION OF CH_2FH_4[b]

(Values = Ave. ± S.D.)

	5-FU Alone		5-FU + CH_2FH_4	
Hours	Free TS[c] (pmol/g)	% Inhibition	Free TS[c] (pmol/g)	% Inhibition
1	1.67 ±0.28	83.3 ±2.8	0.41 ±0.26	95.9 ±2.6
			0.164 ±0.13	98.4 ±1.3
3	1.00 ±0.72	90.0 ±7.2	0.36 ±0.06	96.4
			0.71 ±0.03	92.9
6	1.27 ±0.06	87.3 ±0.6	0.46 ±0.05	95.4

[a] 80 mg/kg i/p.

[b] 27 mg/kg in (6R) CH_2FH_4 by spectrophometric and binding assays.

[c] Not corrected for ternary complex exchange labeling or ratio of CH_2FH_4 to FH_4. A minimal correction factor of 5% leads to the calculation that there was 100% TS inhibition for all tumors receiving the combination of 5-FU and CH_2FH_4, compared to only 92% average TS inhibition by 5-FU alone. Baseline total TS was 10.00 ± 0.04 pmol/g.

Example 3

CH_2FH_4 was formulated, assayed, and administered to 2 patients who had previously been treated with 5-FU. The assays were performed by the methods described in Spears et al., Adv. Exp. Med. Biol. 244:98-104 (1988). In the data shown, the TS inhibition profiles that resulted from CH_2FH_4 administration were not due to concurrent 5-FU dosing. The most recent exposure to 5-FU in these cases was slightly greater than a week prior to the study date, with the patients eligible, however, from the standpoint of toxicity evaluation to receive the weekly dose of 5-FU. Thus, residual FdUMP levels from previous exposure, below the detectable limits for assay, were expected to be present (See Spears et al. Mol. Pharmacol. 27:302-07 (1985)). The serial biopsies were done following single dose administration of CH_2FH_4.

The formulation of CH_2FH_4 was as described in Example 2, and was performed on the day of CH_2FH_4 administration. The assays were also performed on the day of CH_2FH_4 administration.

The results in these patients of the pharmacodynamic tumor tissue analyses showed striking evidence of TS inhibition following CH_2FH_4 administration. These results are summarized in Tables II and III below.

TABLE II

TS INHIBITION AFTER CH_2FH_4 ADMINISTRATION

PATIENT:	A.M.; last 5-FU treatment: ≥ 1 week
LOCATION:	Östra Sjukhuset (Eastern Hospital), Sweden
TUMOR:	Skin metastasis from gastric carcinoma
CH_2FH_4 FORMULATION:	0.1 M Na Ascorbate, pH <9.5, Sigma (6R,S)CH_2FH_4, DEAE-column purified
CH_2FH_4 DOSE:	30 mg in 30 cc IV over 2 min; 4 mg as parent CH_2FH_4, 26 mg as FH_4.

(Tumor Tissue Values = Ave. ± S.D.)

Time of Biopsy[a]	THYMIDYLATE SYNTHASE (TS)[b] pmol/g	% of Baseline	FBC[c] nmol/g	% of Baseline
0 min	1.31 ±0.13	(100)	5.88 ±0.56	(100)
10 min	0.26 ±0.17	19.8	0.23 ±0.02	3.9
20 min	0.56 ±0.06	42.7	0.27 ±0.01	4.6
40 min	0.99 ±0.08	75.6	0.21	3.6
60 min	1.47 ±0.13	112.2	0.14 ±0.01	2.3

[a] Biopsies of solitary skin metastasis, average weight 68 ± 58 mg, time after CH_2FH_4 administration.

[b] By [6-^{3}H]FdUMP ligand-binding assay (CP Spears et al., Cancer Res. 42:450-56 (1982).

[c] Folate Binding Capacity, FBC, is a measure of tissue CH_2FH_4 and FH_4 level (Invest. New Drugs 7:27-36 (1989), (modified after Priest et al., Biochem. J. 216:295-98 (1983)), with a Sigma (6R,S)-CH_2FH_4 standard value of 936 DPM/pmole.

TABLE III

TS INHIBITION AFTER CH_2FH_4 ADMINISTRATION

PATIENT:	K.H.; last 5-FU treatment: ≥ 1 week
LOCATION:	Östra Sjukhuset (Eastern Hospital), Sweden
TUMOR:	Rectal adenocarcinoma, locally advanced
CH_2FH_4 FORMULATION[a]:	0.2 M Na Ascorbate, Sigma (6R,S)-CH_2FH_4
CH_2FH_4 DOSE:	35 mg IV over 1 min week #1; 50 mg IV in 40 ml week #2

(Tumor Tissue Values = Ave. ± S.D.)

Time of Biopsy[b]	THYMIDYLATE SYNTHASE (TS)[c] pmol/g Week #1	% of Baseline Week #2	FBC[d] ΔDPM Week #1	% of Baseline Week #2
0 min	5.77 (100) ±0.09	5.64 (100) ±1.26	759 (100) ±145	499 (100) ±190
10 min	6.28 (212.4) ±1.92	10.25 (181.7) ±0.82	320 (42.2) ±60	376 (75.4) ±17
20 min	2.26 (43.7) ±0.36	5.91 (104.8) ±0.17	314 (41.4) ±9	814 (163.1)
30 min	5.90 (114.1) ±0.12	2.02 (35.8) ±0.03	632 (83.3) ±26	249 (49.9) ±75
40 min		3.46 (61.3) ±0.28		399 (80.0) ±44
24 hr	6.32 (122.2) ±0.52		1403 (184.8) ±130	

[a] On Week #1 the CH_2FH_4 was formulated at pH 2.0, DEAE-purified; On Week #2 the preparation was pH 9.0, with 6 mM (final concentration) CH_2O added, no DEAE step used.

[b] Biopsies of rectal pouch mass, average weights, 145 ± 39 mg (Week #1) and 136 ± 24 mg (Week #2). Time after CH_2FH_4 administration.

[c] By [6-^{3}H]FdUMP ligand-binding assay (Spears et al., Cancer Res. 42:450-56 (1982)).

[d] Folate Binding Capacity, given in ΔDPM over [^{3}H]FdUMP-TS binary complex background (Invest. New Drugs 7:27-36 (1989)); standard curve Sigma (6R,S)-CH_2FH_4 showed 920 and 898 ΔDPM/pmole for weeks 1 and 2. Multiply ΔDPM values by 0.0002 to convert to nmol/g.

In patient A.M., a sixty-seven year old woman with over a 3 year prior history of disseminated gastric cancer, and who was end-stage in her course, TS was inhibited 80.1 and 57.3 % in her tumor at 10 and 20 min, respectively, in her tumor after CH_2FH_4 administration. (It should be noted that the CH_2FH_4 preparation was over 85% FH_4.) Notably, when she was studied again 2 weeks subsequently, with a repeat dose of CH_2FH_4, TS in the baseline tumor biopsy was undetectable (data not shown).

The FBC (folate binding capacity of L. casei TS-[3H]FdUMP added to the cytosols, (a measure of tissue CH_2FH_4 and FH_4, mostly presumed to be polyglutamates) also showed a surprising decrease, which continued through 60 min. Tissue FH_4 polyglutamates were not separately measured by use of CH20 addition to the FBC conditions. The continuing drop in FBC, however, at the 60-min time point rules out the possibility that all post-CH_2FH_4 biopsies were somehow an artifact of tumor tissue sampling. This paradoxical decrease in FBC is a characteristic feature of 5-FU-responding patients receiving high-dose LV added to 5-FU bolus i.v. therapy (C.P. Spears, et al. Presentation at 25th Annual Am. Soc. Clin. Oncol. meeting, May 22, 1989). This decrease was also seen in tumor of patient K.H. (Table 3). An explanation for the paradoxical decrease in FBC is that one-carbon exchange (e.g., R.G. Matthews et al, Adv.Enz.Regul. 26:157-70 (1987) occurred in the tumor tissue, between FH_4-monoglutamate derived within minutes from administration of the CH_2FH_4/FH_4 drug, and endogenous CH_2FH_4-polyglutamates. Since the polyglutamates of CH_2FH_4 may be expected to bind TS-FdUMP up to 50-fold more strongly than the monoglutamate (Houghton et al., Cancer Res. 48:3062-69 (1988)), the one-

carbon exchange could lead to the observed decrease. This data is powerful evidence that CH_2FH_4/FH_4 given to this patient was rapidly transported and metabolized in her tumor. The decrease in TS in her tumor, then, is assumed to be related to this metabolism and the presence of non-measurable levels of FdUMP (at concentrations near stoichiometry with endogenous TS binding sites). The paradox of decreasing free TS with decreasing FBC also can be explained by metabolic channeling of administered CH_2FH_4 (Reddy et al., Proc. Natl. Acad. Sci. USA 77:3312-16, 1980), or by formation of TS-FdUMP-tetrahydrofolate, or of TS-deoxyuridylate-CH_2FH_4 ternary complexes by the unnatural (6S)-CH_2FH4 or (6R)-FH_4 enantiomer, or by TS-FdUMP-CH_2FH_4 due to very rapid ternary complex formation (Lockshin et al., Biochem. Pharmacol. 30:247-57 (1981)) prior to the 10-min biopsy sample and one-carbon folate metabolism. In fact, the last explanation may be the most attractive, since the maximum TS inhibition was at this first biopsy time point. The degree of TS inhibition, 80.2% decrease over baseline value, and relatively limited duration of TS inhibition would predict that higher concentrations of FdUMP (as would result from 5-FU given shortly before, or with the CH_2FH_4) would lead to the desired therapeutic objective of complete TS inhibition.

In patient K.H., a fifty-five year old man with locally unresectable advanced rectal adenocarcinoma, the TS pharmacodynamic tumor tissue analyses were done twice, nine days apart. Following study, K.H. continued to receive intermittent bolus 5-FU. This patient had been previously a partial responder to 5-FU plus LV, with stable disease at the time of initial CH_2FH_4 administration. There were modifications of the CH_2FH_4 formulation between the 2 pharmacodynamic

studies (See Table III). In the first study week, the pH was not adjusted up from 2.0, after DEAE column isolation of the Sigma (6R,S)-CH_2FH_4. Thus, some of this folate may also have been 5,10-methenyl-tetrahydrofolate. In the second study week, the pH was adjusted up to 9.0, and no DEAE step was used (with therefore 6 mM formaldehyde being present in the 40-cc volume for injection).

Patient K.H. showed changes in TS and in FBC assays after CH_2FH_4 administration that were qualitatively similar to those of Patient A.M., shown in Table III. Again, significant inhibition of TS over baseline values occurred in tumor samples after the CH_2FH_4 was given, in the absence of recent 5-FU exposure. On the first occasion, however, the pH of the formulation was low, and possibly the CH_2FH_4 was less well solubilized (or less stable, or both) than on Week #2, when an alkaline pH was used in addition to an excess of CH_2O. Comparison with patient A.M. suggests that the acute TS decrease resulted from FH_4 rather than CH_2FH_4. As in Patient A.M., TS inhibition, on both occasions, was transient, averaging 36 to 44% of baseline values for the combined data of the two studies, during the 20 to 30 min period after CH_2FH_4 was given. The most significant evidence of an increase in CH_2FH_4, as reflected by FBC assay, was at 24 hr after the first dose, which was expected on the basis of slow polyglutamation of folates generally. Significant drops in FBC also occurred in both weeks of study, again suggestive of the postulated one-carbon exchange between drug-monoglutamates and endogenous CH_2FH_4-polyglutamates. The fact of a less striking change in FBC values in tumor biopsies from K.H. than in A.M. is also consistent with the lower baseline FBC values (given in raw DPM, multiply by 0.0002 to convert to nmol/g units comparable to

Patient A.M.), and the less striking but highly significant TS inhibition in tumor of K.H. As with Patient A.M., the data would predict, using purely kinetic arguments, that higher FdUMP levels generated from 5-FU given closer to the time of CH_2FH_4 dosing would lead to desired abrogation of TS activity.

It has long been known that FdUMP tends to persist at low levels in tissues following a single dose of 5-FU. FdUMP may therefore be slowly released from the RNA storage compartment inside cells.

Thus, because only trace concentrations of FdUMP are required to inhibit TS, if CH_2FH_4 or FH_4 levels are high, the TS inhibition observed in these two patients was likely to have been due to facilitation by the natural (6R)-CH_2FH_4 or (6S)-FH_4 enantiomers (diastereomers) of the CH_2FH_4 formulation on TS binding by residual FdUMP levels. These results suggest that repeated administration of CH_2FH_4 or FH_4 may be as effective as repeated dosing with 5-FU, but without the toxicity of dose-escalation of 5-FU.

The patients who received CH_2FH_4 showed no acute toxicities due to this treatment, including the instance of week #2 in K.H. when a slight excess of CH_2O was present in the preparation. However, they did continue to manifest the same toxicities as their prior experience with 5-FU plus LV (i.e., mild nausea and fatigue). Patient A.M., as noted above, had extremely advanced gastric cancer at the time of the study and so was not evaluable for response. However, patient K.H. showed endoscopic evidence of continued disease stabilization if not at least additional, minor tumor regression noted over the subsequent months after the two weeks of CH_2FH_4 administration.

Example 4

(6R,S)-FH$_4$ ADMINISTRATION TO RATS BEARING TRANSPLANTED HEPATIC COLONIC CARCINOMAS

Table IV (below) shows the results of (6R,S)-FH$_4$ (see Figure 3) administration to rats bearing transplanted hepatic colonic carcinoma. The present inventors have considerable experience with this model, and the antitumor effects of 5-FU shown are typical results, as are the TS and folate assays of control and 5-FU-only-treated rats. A striking finding was of growth stimulation yet decreased TS levels after (6R,S)-FH$_4$ alone. In fact, the"free TS" levels in the (6R,S)-FH$_4$-only-treated rats were the lowest of all arms of the study. This observation suggests that either the natural 6S-FH$_4$ or the unnatural 6R-FH$_4$ may have formed TS-inhibitory TS-dUMP-folate ternary complexes. In combination, the degree of synergy of (6R,S)-FH$_4$ with 5-FU in this example appears to be greater than previously found for (6R,S)-leucovorin (Carlsson et al., Anticancer Res. 10:813-16 (1990)).

TABLE IV

(6R,S)-TETRAHYDROFOLATE[a] AS A MODULATOR OF 5-FU IN AN EXPERIMENTAL LIVER CANCER IN RATS[b]

RESULTS AT DAY 17 AFTER TRANSPLANTATION
(Average of 3 rats/treatment)

TREATMENT	TUMOR WEIGHT (g)	TS[d] (p mole/g)	5,10-CH$_2$FH$_4$[e] (nmol/g)	FH$_4$[e] (nmol/g)
CONTROL	5.84	18.96	0.69	1.18
5-FU ONLY (30 MG/KG)	1.03	9.03	4.11	2.39
5-FU[c] + (6R,S)-FH$_4$[c]	0.31	9.23	1.23	1.76
(6R,S)-FH$_4$ only (30 mg/kg)	10.43	7.13	2.93	2.31

[a] (6R,S)-FH_4 was the commercially available racemic tetrahydrofolate from Fluka Chemical Corp. (Cat. No. 87355, "Tetrahydrofolic acid dihydrochloride monohydrate," or "5,6,7,8-Tetrahydropteroyl-L-glutamic aicd dihydrochloride monohydrate," >94% by HPLC). The (6 R,S)-FH_4 was weighed, dissolved in normal saline, and injected Days 2-5 by tail vein administration using the air-free Protector device to prevent oxidative destruction of the folate.

[b] Inoculation of 1 x 10^6 viable colon tumor (nitrosoguanidine-induced) cells under the liver capsule on Day 1 (Carlsson et al., Anticancer Res. 10:813-16 (1990)). Animals sacrificed on Day 17 for excision of single liver tumor nodules for pharmacodynamic studies.

[c] 30 mg/kg

[d] Assays done as desribed (Spears et al. Adv. Exp. Med. Biol. 244:98-104 (1988)) and done at 24 h after injection.

Example 5

Spontaneous Conversion of CH_2FH_4 to FH_4 by Dilution

Figure 4 shows the results of TS-[^{3}H]FdUMP-folate binding assay of CH_2FH_4 as a function of concentration of the folate in 0.2 M Tris burffer, pH 7.4, with and with formaldehyde (CH_2O), 6 mM, addition. The CH_2FH_4 was prepared as the racemic (6R,S) material from (6R,S)-FH_4 and excess formaldehyde, and DEAE-column isolation as described in Figure 1. This preparation was essentially free of free formaldehyde based on colorimetric assay of bulk material (Nash, Biochem. J. 55:416-21 (1953)).

At all concentrations (total assays volume 150 μl), excess formaldeyde was required to obtain maximal binding (which was still only 19.3% of stoichiometric binding). A notable effect was the increasing need for formaldehyde addition with increasing dilution, to obtain maximal CH_2FH_4 assay recovery.

This phenomenon has been a repeated observation in the laboratories of the inventors, and clearly shows that CH_2FH_4 on dilution becomes FH_4 with liberation of free formaldehyde. The concentration requirement for formaldehyde to reverse the FH_4 formation caused by dilution is in the millimolar range which is vastly higher than physiologic.

This requirement for a large excess of formaldehyde to shift the equilibrium between FH_4 and CH_2FH_4 (Eq. 1) was found by the inventors to

$$CH_2FH_4 = FH_4 + CH_2O \quad \text{Eq. 1}$$

be independent of temperature, pH or formaldehyde content of charcoal isolation, the presence of air exposure, or the presence of reducing agents. In addition, [11-^{14}C]CH_2FH_4 prepared as described (Moran et al., Proc. Natl. Acad. Sci. USA 76:1456-60 (1979)), and DEAE-purified (as the concentrated material) of excess $^{14}CH_2O$, was confirmed to have a labile $^{14}CH2O$ group by dimedone trapping. For instance, 46,664 DPM of [11-^{14}C]-CH_2FH_4 diluted to 1 ml in H_2O was found to have 67.8% of the label recoverable by chloroform extraction of dimedone (methone) product (37°C).

CLAIMS:

1. A method of inhibiting the growth of a tumor in a patient comprising administering to said patient an amount of 5,10-methylene-tetrahydrofolate (CH_2FH_4) and 5-Fluorouracil (5-FU) sufficient to effect said growth inhibition.

2. The method of claim 1 wherein CH_2FH_4 is administered to said patient concurrently with 5-FU.

3. The method of claim 1 wherein CH_2FH_4 is administered to said patient prior to the administration of 5-FU.

4. The method of claim 3 wherein CH_2FH_4 is administered to said patient 6-24 hours prior to the administration of 5-FU.

5. The method of claim 4 wherein CH_2FH_4 is administered to said patient 1-3 hours prior to the administration of 5-FU.

6. The method of claim 1 wherein CH_2FH_4 is administered to said patient subsequent to the administration of 5-FU.

7. The method of claim 6 wherein CH_2FH_4 is administered to said patient 1-10 days subsequent to the administration of 5-FU.

8. The method of claim 7 wherein CH_2FH_4 is administered to said patient 1-6 hours subsequent to the administration of 5-FU.

9. The method of claim 1 wherein CH_2FH_4 is administered to said patient intravenously, intraarterially or intraperitoneally.

10. The method of claim 9 wherein CH_2FH_4 is administered in a dosage of 5-500 mg/m^2.

11. The method of claim 10 wherein CH_2FH_4 is administered in a dosage of 20-200 mg/m^2.

12. The method of claim 10 wherein CH_2FH_4 is administered intravenously.

13. The method of claim 12 wherein CH_2FH_4 is administered to said patient every 4-6 hours.

14. The method of claim 12 wherein CH_2FH_4 is administered to said patient once daily.

15. The method of claim 12 wherein CH_2FH_4 is administered to said patient once weekly.

16. The method of claim 13 wherein CH_2FH_4 is administered prior to the administration of 5-FU.

17. The method of claim 13 wherein CH_2FH_4 is administered subsequent to the administration of 5-FU.

18. The method of claim 14 wherein CH_2FH_4 is administered to said patient through a central venous catheter.

19. The method of claim 1 wherein CH_2FH_4 is administered to said patient as the 6R diastereomer, the 6S diastereomer, or a mixture of the 6R and 6S diastereomers.

20. The method of reducing toxicity of an anti-folate drug in a patient administered said drug comprising administering to said patient an amount of CH_2FH_4 sufficient to reduce said toxicity.

21. The method of claim 20 wherein the anti-folate drug is methotrexate, trimetrexate, nitrous oxide or dideoxytetrahydrofolic acid.

22. A method of treating folate deficiency comprising administering to a patient in need of such treatment an amount of CH_2FH_4 sufficient to effect said treatment.

23. The method of claim 1 wherein the concentration of CH_2FH_4 administered is from 0.1 to 20 mg/ml in alkaline vehicles.

24. The method of claim 1 wherein the concentration of CH_2FH_4 administered is from 0.1 to 10 mg/ml in physiologic saline.

25. A method of treating B12- and B6-refractory anemias comprising administering to a patient in need of such treatment an amount of CH_2FH_4 sufficient to effect said treatment.

26. A composition comprising an amount of CH_2FH_4 and 5-FU sufficient to inhibit tumor growth in a patient together with a pharmaceutically active carrier.

27. The composition of claim 26 further comprising an agent that stabilizes CH_2FH_4.

28. The composition of claim 27 wherein the agent that stabilizes CH_2FH_4 is an ascorbate salt.

29. The composition of claim 27 wherein the agent that stabilizes CH_2FH_4 is reduced glutathione.

30. The composition of claim 26 further comprising formaldehyde.

31. A composition comprising an amount of CH_2FH_4 and a drug which is metabolized to fluorodeoxyuridylate (FdUMP) sufficient to inhibit tumor growth in a patient together with a pharmaceutically active carrier.

32. The composition of claim 31 wherein the drug which is metabolized to FdUMP is floxuridine (FUDR), ftorafur, or 5'-deoxyfluorouridine.

33. The method of claim 9 wherein CH_2FH_4 is administered to said patient by protracted, continuous venous infusion through a central venous catheter.

34. A method of inhibiting the growth of a tumor in a patient comprising administering to said patient an amount of tetrahydrofolate (FH_4) and 5-Fluorouracil (5-FU) sufficient to effect said growth inhibition.

35. The method of claim 34 wherein FH_4 is administered to said patient concurrently with 5-FU.

36. The method of claim 34 wherein FH_4 is administered to said patient prior to the administration of 5-FU.

37. The method of claim 36 wherein FH_4 is administered to said patient 6-24 hours prior to the administration of 5-FU.

38. The method of claim 37 wherein FH_4 is administered to said patient 1-3 hours prior to the administration of 5-FU.

39. The method of claim 34 wherein FH_4 is administered to said patient subsequent to the administration of 5-FU.

40. The method of claim 39 wherein FH_4 is administered to said patient 1-10 days subsequent to the administration of 5-FU.

41. The method of claim 40 wherein FH_4 is administered to said patient 1-6 hours subsequent to the administration of 5-FU.

42. The method of claim 34 wherein FH_4 is administered to said patient intravenously, intraarterially or intraperitoneally.

43. The method of claim 42 wherein FH_4 is administered in a dosage of 5-500 mg/m^2.

44. The method of claim 43 wherein FH_4 is administered in a dosage of 20-200 mg/m^2.

45. The method of claim 43 wherein FH_4 is administered intravenously.

46. The method of claim 45 wherein FH_4 is administered to said patient every 4-6 hours.

47. The method of claim 45 wherein FH_4 is administered to said patient once daily.

48. The method of claim 45 wherein FH_4 is administered to said patient once weekly.

49. The method of claim 46 wherein FH, is administered prior to the administration of 5-FU.

50. The method of claim 46 wherein FH, is administered subsequent to the administration of 5-FU.

51. The method of claim 47 wherein FH, is administered to said patient through a central venous catheter.

52. The method of claim 34 wherein FH, is administered to said patient as the unnatural 6R diastereomer, the natural 6S diastereomer, or a mixture of the 6R and 6S diastereomers.

53. The method of claim 34 wherein the concentration of FH, administered is from 0.1 to 20 mg/ml in alkaline vehicles.

54. The method of claim 34 wherein the concentration of FH, administered is from 0.1 to 10 mg/ml in physiologic saline.

55. A composition comprising an amount of FH, and 5-FU sufficient to inhibit tumor growth in a patient together with a pharmaceutically active carrier.

56. The composition of claim 55 further comprising an agent that stabilizes FH_4.

57. The composition of claim 56 wherein the agent that stabilizes FH_4 is an ascorbate salt.

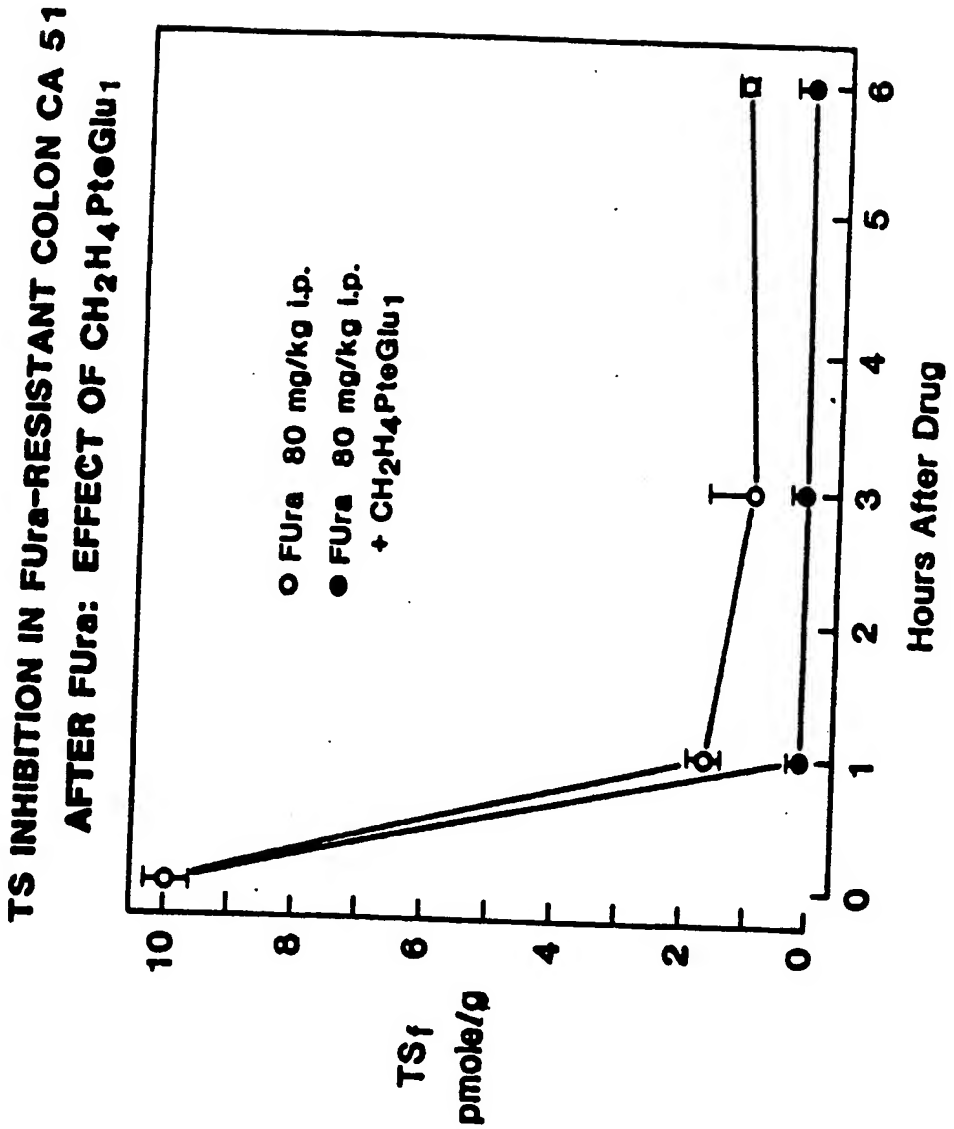
58. The composition of claim 56 wherein the agent that stabilizes FH_4 is reduced glutathione.

59. The composition of claim 56 wherein the agent that stabilizes FH_4 is formaldehyde.

60. A composition comprising an amount of FH_4 and a drug which is metabolized to fluorodeoxyuridylate (FdUMP) sufficient to inhibit tumor growth in a patient together with a pharmaceutically active carrier.

61. The composition of claim 60 wherein the drug which is metabolized to FdUMP is floxuridine (FUDR), ftorafur, or 5'-deoxyfluorouridine.

62. The method of claim 34 wherein FH_4 is administered to said patient by protracted, continuous venous infusion through a central venous catheter.


TS INHIBITION IN FUra-RESISTANT COLON CA 51
AFTER FUra: EFFECT OF $CH_2H_4PteGlu_1$
O FUra 80 mg/kg i.p.
● FUra 80 mg/kg i.p.
+ $CH_2H_4PteGlu_1$
TS_f pmole/g
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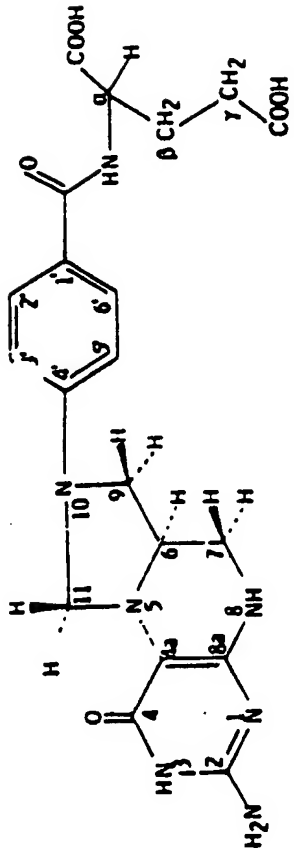
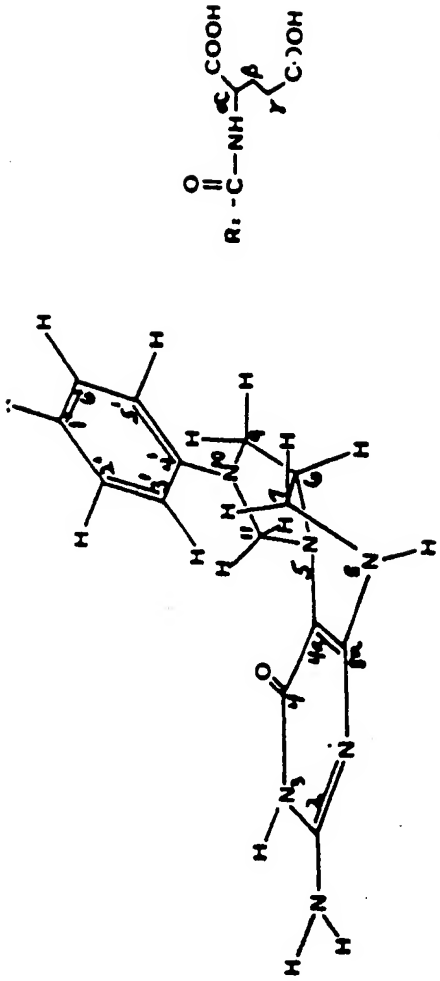
2
4
6
8
10
0
1
2
3
4
5
6
Hours After Drug

Fig. 1

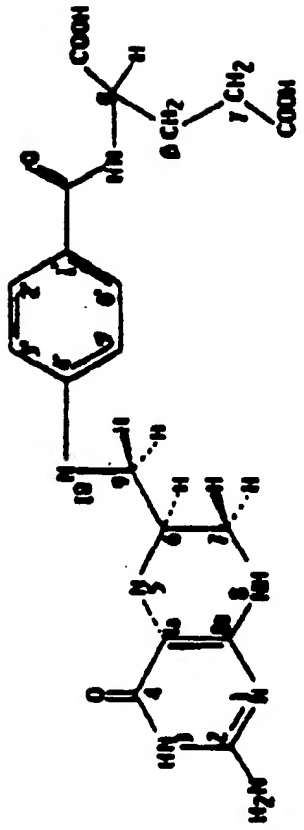


(6R,S)-methylene-tetrahydrofolic acid or CH2FH4



Configuration of the natural (6R)-CH2FH4 enantiomer

Fig 2



tetrahydrofolic acid or FH4

Fig. 3

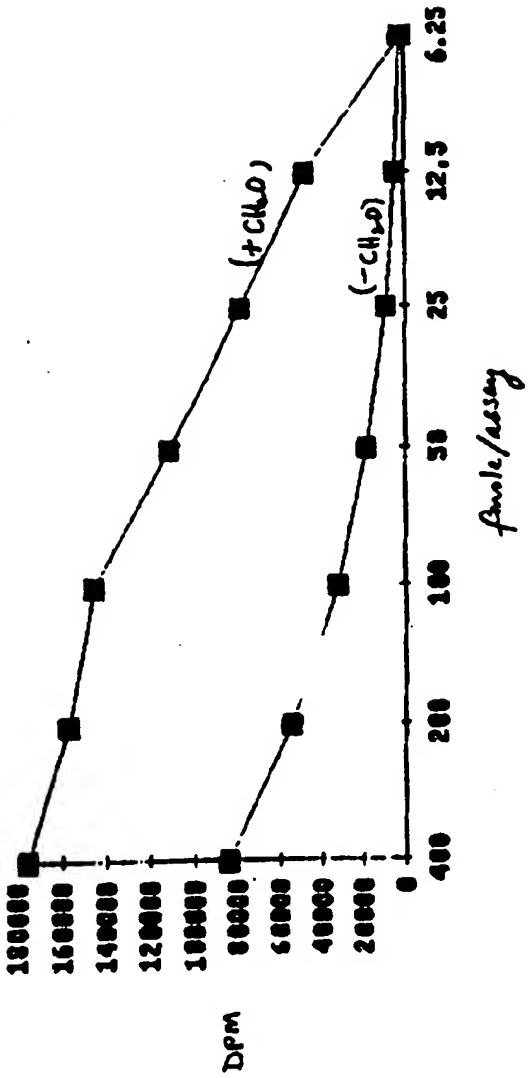


Fig. 4

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/03186

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A01N 431/54
US : 514/274

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	514/274

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

CAS ON LINE: A.P.S.

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X,Y	Spears, et al, Method for Thymidylate-Synthase Pharmacodynamics: Serial Biopsy, Free and total T,S Fdump, and H_4 PTEGLUM and CH_2-H, PTEGLU Assays; Adv. Exp. Med. Biol.; 244:98-104(1988) See entire document	1-62
Y	Spears, et al Activation of Leucovorin (CF) To Methylenetetre hydroflolate (CH_2FH_4) for Improving Thymidylate Synthase (TS) Inhibition after 5-F4: Effects of CF Dose, L-Serine, L-Glutamate, and direct Methyl-Tetrahydro-folate (CH_3FH_4) Administration, Proceedings of Asco, Vol. 8, March 1989 (#269), pg. 69. See entire document	1-62
Y	Grem et al, Overview of Current Status and Future Director of Clinical Trials with 5-Fluorouracil in combination with Folinic Acid, Cancer Treatment Reports, Vol 71, No. 12, December, 1987 Pgs. 1249-1264. See entire document	
Y	Machorer, et al, Treatment of Advanced Colorectal and Gastric Adrenocarcinomas with 5-FM combined with High-Dose Folinic Acid: A Pilot study, Cancer Treatment	1-62

(Cont. on second sheet)

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
13 August 1991	26 AUG 1991
International Searching Authority	**Signature of Authorized Officer** [14]
ISA/US	Theodore J. Criares

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	(Cont. from 2nd sheet) Reports, Vol. 66, No 10, October, 1982, Pgs 1803-1807. See entire document	